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An antibacterial and antifungal phenylpropanoid from *Carum montanum* (Coss. et Dur.) Benth. et Hook.

Hocine Laouer[1], El Kolli Meriem[1], Soizic Prado[2], and Nicolas Baldovini[3,*]

[1]Department of Biology, University of Setif, Mabouda, 19000, Setif, Algeria

[2]Laboratoire de Chimie et Biochimie des Substances Naturelles - UMR 5154 CNRS, Muséum National d'Histoire Naturelle, 63 rue Buffon, 75005 Paris, France.

[3]Université de Nice Sophia-Antipolis, Laboratoire de Chimie des Molécules Bioactives et des Arômes, CNRS UMR 6001, Parc Valrose, F-06108 Nice cedex 2, France.

The volatile constituents of the aerial parts of *Carum montanum* (Coss. et Dur.) Benth. et Hook. were analysed by GC-FID and GC-MS, and the main component was isolated and identified as nothoapiole. The antibacterial and antifungal activities of this compound and of the total oil were investigated against Gram negative (*P. aeruginosa, E. coli*), Gram positive (*E. faecalis, S. aureus*, *S. epidermitis, S. saprophyticus, S. simulans, S. lugdunensis*) bacteria and on one strain of fungus (*C. tropicalis*).

Keywords : Carum montanum; Apiaceae ; essential oil; nothoapiole; phenylpropanoids; antibacterial activity; antifungal activity.

*Correspondence to : Dr. N. Baldovini, Université de Nice Sophia-Antipolis, LCMBA, CNRS UMR 6001, Parc Valrose, F-06108 Nice cedex 2, France.

E-mail : baldovin@unice.fr

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INTRODUCTION

Caraway (*Carum carvi* L.) is one of the oldest spices cultivated in Europe. The fruit of caraway is a schizocarp, which at harvest splits into two halves, erroneously called ‘‘seeds’’, which are used in the flavouring of bread (e.g., rye bread), cheeses, sauerkraut, candies, meat products, sauces and alcoholic liqueurs, such as the German Kummel. It is also a source of carvone for cosmetics, toothpaste, chewing gum and pharmaceutical preparations. The seeds have been used in alternative medicine for the treatment of colics, loss of appetite and digestive disorders as well as to dispel worms. Antipasmodic, emmenagogue, expectorant, galactogogue, stimulant, stomachic and tonic properties have also been reported (de Carvalho and da Fonseca, 2005).

While *Carum carvi* L. is the best known member of the *Carum* genus (Apiaceae), several other species have been investigated regarding their chemical composition and biological properties, especially *C. copticum* (Boskabady *et al.*, 2003; Dashti-Rahmatabadi *et al.*, 2007; Sahaf *et al.*, 2007) and to a lesser extent *Carum nigrum* (Singh *et al.*, 2006).

In the course of our studies of the pharmacological properties of endemic plants from Algeria, we investigated the phytochemistry of a little-known species of the *Carum* genus: *C. montanum* (Coss. et Dur.) Benth. et Hook. [syn. *Selinopsis montana* Coss. et Dur.], which is one of the two endemic *Carum* species described in Algeria (Quezel and Santa, 1963), the other one beeing *C. foetidum* (Coss. et Dur.) Benth. et Hook. syn. *Selinopsis foetida* Coss. et Dur. *C. montanum* grows wild in calcareous mountainous regions, such as the Constantine Saharian and Tell Atlas, as well as in the Kabyle and

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Numidian areas. It is characterized by smooth stems (10 - 15 cm), white flowers and an oblong fruit (2 - 2.6 mm) (Quezel et santa, 1963). Contrary to *C. carvi*, this species has neither medical nor culinary applications, but is commonly grazed by the livestock.

A single study was published on the chemistry of *C. montanum*, in which xanthotoxin and a new furanocoumarin glycoside were identified in the aerial parts (Benahmed *et al.*, 2006). To investigate other secondary metabolites of this species, we analysed the volatile components of the roots and the aerial parts, as well as their antimicrobial effect against various Gram negative and Gram positive bacteria and on one strain of fungus. We evaluated also the activity of the major constituent, nothoapiole, isolated from the mixture of volatiles.

MATERIAL AND METHODS

Plant material. Fully flowered aerial parts of *Carum montanum* (Coss. et Dur.) Benth. et Hook. were collected in Megress Mountain, at 1500 m above sea level, during May 2006. A voucher specimen (B-6306) has been deposited in the Muséum d'Histoire Naturelle de la Ville de Nice, France.

Extraction of volatiles and isolation of nothoapiole. Essential oil was obtained by hydrodistillation of dried aerial parts (1.2 ml/kg : 1.1% yield) or roots (0.8 ml/kg : 0.7% yield) using a Clevenger type apparatus, and was dried over anhydrous sodium sulfate. Pure nothoapiole was obtained by several successive column chromatographies on silica gel using petroleum ether/diethyl ether gradients for elution.

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General. The NMR spectra were recorded on a Bruker WM 200 or 500 MHz spectrometer in $CDCl_3$. The chemical shift values are reported with reference to TMS and the coupling constants are given in Hz. GC and GC-MS analyses were carried out using an Agilent 6890N gas chromatograph apparatus equipped with a flame ionization detector (FID) and coupled to a quadrupole Agilent 5973 network mass selective detector working in electron impact (EI) mode at 70 eV (scanning over 35-350 amu range). The gas chromatograph was equipped with two fused silica capillary columns HP-1 (PDMS, 50 m × 0.2 mm i.d., film thickness : 0.33 µm). The analytical parameters (identical for GC and GC-MS analyses unless specified) were the following: The carrier gas was helium at a flow rate of 1 mL/min. The oven temperature was programmed from 60 to 250°C at 2°C/min and held isothermal for 40 min. The injector (split mode, ratio 1/100) temperature was 250°C. The FID temperature was set at 250°C, and in the GC-MS analyses, the temperatures of the ion source and transfer line were 170 and 280°C, respectively. The constituents of the essential oil were identified by comparison of their mass spectral pattern and retention indices (RI) with those of pure compounds registered in commercial libraries and literature data (McLafferty and Staufer, 1989; BACIS, 1999; Joulain and König, 1999; NIST, 1999) and with a laboratory-made database built from authentic compounds.

Bacterial strains and cultures conditions. References strains of *Staphylococcus aureus* subsp. *aureus* (ATCC 6538), *Staphylococcus epidermitis* (CIP 10464), *Staphylococcus saprophyticus* subsp. *Saprophyticus* (CIP 10464), *Staphylococcus simulans* (CIP 81.64), *Staphylococcus lugdunensis* (CIP 103584) were obtained from the Collection of the Institut Pasteur, Paris, France. *Enterococcus faecalis* (14C1104)

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and *Pseudomonas aeruginosa* (13C3104) strains were isolated from patients and kindly provided by Laurent Marsollier (Institut Pasteur). *Escherichia coli* (ATCC 9738) and *Candida tropicalis* (ATCC 66029) were from the National Museum of Natural History (NMNH). Bacteria species were cultivated for 24 hrs in Mueller Huntington's medium (MH) at 37°C and for 48 hrs at 30°C in Sabouraud dextrose medium (Sanofi Diagnostic Pasteur) for *C. tropicalis.*

Disc diffusion assay. The antibacterial activity of the essential oil was evaluated using the standardized filter paper disk (6 mm non impregnated disk. Antibiotica assay discs, Grade 2668 Schleider and Schuell) diffusion method according to the Kirby-Bauer method (Bauer *et al.*, 1966). Briefly, culture suspension of the tested microorganisms (approximately 10^6 CFU/mL) was spread on the solid media plates (50 mL). Filter paper discs were impregnated with 10 µL of serial dilutions in dimethylsulfoxide (DMSO, Sigma) of the essential oil and placed onto the solid media plates. The diameter of inhibition was measured after 24 or 48 h of incubation at 30°C or 37°C. Ampicillin (Sigma) and DMSO were used as positive and negative controls, respectively.

RESULTS AND DISCUSSION

Our preliminary investigation of the GC-MS profile of the aerial part essential oil showed a main (62.8%) unidentified constituent, together with common monoterpenes and sesquiterpenes (table 1). By successive preparative column chromatographies on silica gel, this component was isolated in pure form and identified as nothoapiole (**1**) by MS and NMR analysis, and comparison with spectral data reported in the literature

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(Rahman *et al.*, 1999). This highly oxygenated phenylpropanoid is structurally related to myristicin (**2**), apiole (**3**) and dill-apiole (**4**) which are widespread in many vegetal species, but **1** is actually much less common than these compounds: Up to now, **1** was identified in *Carum nigrum* essential oil and oleoresin (Singh *et al.*, 2006) as well as in five other vegetal species : *Perilla frutescens* (Ito *et al.*, 1999), *Pimpinella serbica* (Ivanic *et al.*, 1983), *Molopospermum peloponnesiacum* (Kubeczka and Ullmann, 1983), *Peucedanum pauciradiatum* (Bagirov *et al.*, 1982) and *Nothosmyrnium japonicum* (Saiki *et al.*, 1970) and in the brown alga *Spatoglossum variabile* (Rahman *et al.*, 1999). However, none of these sources contained such a high percentage of nothoapiole as in *C. montanum*. Moreover, although the ^{1}H NMR spectrum of this compound was described several times (Saiki *et al.*, 1970; Bagirov *et al.*, 1982; Rahman *et al.*, 1999), the ^{13}C NMR data with the assignments have never been reported yet, hence these informations were deduced from a series of 1D and 2D NMR experiments including COSY, NOESY, HSQC and HMBC, and are summarised in table 2.

Interestingly, myristicin (**2**), apiole (**3**) and dillapiole (**4**) were also identified in the essential oil of the aerial parts of *C. montanum*, as well as in the essential oil of the roots which consisted almost exclusively in a mixture of **1** and **4** (78% and 9%, respectively). Their co-occurence is not surprising since Ito *et al.* (1999) showed that in *Perilla frutescens*, nothoapiole is biosynthetised by successive enzymatic methoxylations of myristicin, the biogenetic pathway beeing the following : **2** → **4** → **1**. The phenylpropanoids family is rich in biologically active compounds. Myristicin **2** was shown to be insecticide (Lichtenstein and Casida, 1963; Lichtenstein *et al.*, 1974; Berenbaum and Neal, 1985) and to synergize the activity of synthetic insecticides

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(Lichtenstein *et al.*, 1974; Berenbaum and Neal, 1985). Many other interesting properties of **2** were described, such as hepatoprotective (Morita *et al.*, 2003), antiinflammatory (Ozaki *et al.*, 1989) and CNS depressant (Shin *et al.*, 1988). Several studies reported also that this compound could be a potential chemopreventive compound (Zheng *et al.*, 1992; Ahmad *et al.*, 1997). Apiole (**3**) was less studied, but nevertheless showed also a wealth of biological activities, such as antifungal (Meepagala *et al.*, 2005; Razzaghi-Abyaneh *et al.*, 2007), antioxydant (Zhang *et al.*, 2006), phytotoxic (Meepagala *et al.*, 2005) and an even more potent synergistic insecticide potential than **2** (Lichtenstein *et al.*, 1974). In fact, in most of the studies where **2** and **3** or **4** were compared regarding their biological activities, **3** and **4** were more potent than **2** (Lichtenstein *et al.*, 1974; Zhang *et al.*, 2006; Razzaghi-Abyaneh *et al.*, 2007), while unwanted carcinogenic and genotoxic properties (well established for safrole and estragole) decreased with further methoxylations of the aromatic nucleus in the order : safrole > **2** > **3** > **4** (Zhou *et al.*, 2007).

Only one study explored recently the biological activity of a nothoapiole containing mixture through the evaluation of the antioxydant, antibacterial and antifungal properties of *Carum nigrum* essential oil and oleoresin (Singh *et al.*, 2006), but this substance was contained in low percentage in these mixtures and was not evaluated alone. The apparent pharmacological potential of the highly oxygenated phenylpropanoids prompted us to better define the biological potential of **1**, of which *C. montanum* could be an interesting source.

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As shown in table 3, the essential oil of *C. montanum* was devoid of significant antimicrobial activity against the Gram – bacteria tested. The essential oil and its half dilution present a weak activity (respectively 6 and 5 mm) against *E. coli* compared to ampicillin control (12 mm). Strains of *P. aeruginosa* were particulary resistant, even to 10 µl of the essential oil, the highest quantity used in this assay. Nevertheless, a more potent effect on Gram + was obtained. We observed inhibition of the crude extract and its half dilutions on *S. aureus* with diameters of inhibition from 11 to 6 mm. Surprisingly, dilutions of the essential oil did not affect the activity as the diameters of inhibition are relatively constant. In contrast, no activity was observed on *E. faecalis*. These observations lead us to investigate others pathogenic strains from *Staphylococcus* genus such as *S. epidermitis*, *S. saprophiticus*, *S. simulans*, *S. lugdunensis*. The level of antibacterial activity was relatively high and constant at all the concentrations used except on *S. saprophyticus* which turned out to be totally resistant to the essential oil. As seen on Table 3, the most sensitive strain is *S. simulans* with a diameter of inhibition for the crude extract of 28 mm. This is relatively close from the one obtained for 30 µg of ampicillin. No significant difference of activity was observed for half dilution, but a loss of activity was measured for the following dilutions. Good activity was also noted on *S. lugdunensis* with a 12 mm diameter of inhibition for the highest concentration tested. This diameter is actually more important than the 9 mm observed for 30 µg of ampicillin. A related range of activity on fungus *C. tropicalis* was observed with a diameter of inhibition around 15 mm at the highest concentration. The lack of effect of ampicillin on *Candida* growth is consistent with this antibacterial agent.

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In a second step, we also evaluated the antibacterial activity of pure nothoapiole (**1**). As observed for the total extract, **1** did not inhibit *E. coli* growth. Moreover, as for the essential oil, a strong antibacterial activity was measured on *Staphylococcus* genus. As shown in table 3, 10 µl of nothoapiole inhibited the growth of *S. epidermitis* and *S. simulans* with a 8 mm diameter of inhibition. Interestingly, this activity, although significant, is actually lower than that of the total essential oil. On the other hand, the inhibition on *S. lugdunensis* and *C. tropicalis* was close to the level of effect exhibited by the essential oil.

In conclusion, the essential oil of *Carum montanum* displayed a good activity against Gram + bacteria, particularly against *Staphylococcus* strains. The results against fungus *C. tropicalis* are also of interest and further studies on other fungi strains will help in the evaluation of the therapeutic potential of this essential oil. The nothoapiole inhibition of *Staphylococcus* strains growth we observed pointed out the contribution of this component to the antibacterial activity of the entire essential oil. In the case of the activity on *C. tropicalis*, the related level of activities observed suggest that nothoapiole is the main compound causing the antifungal effect of *C. montanum* essential oil.

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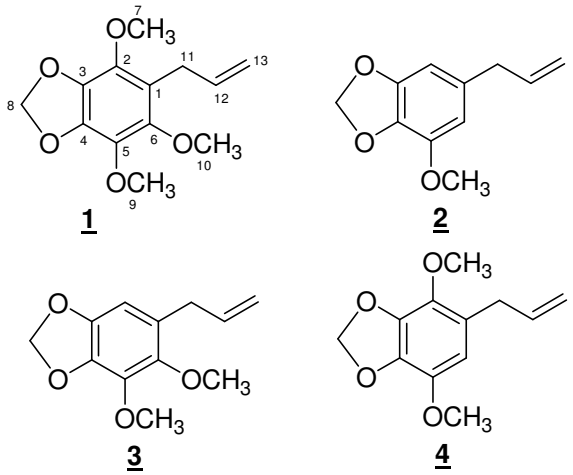

Figure 1. Structure of nothoapiole (**1**) and of selected oxygenated phenylpropanoids.

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Table 1. Composition of the essential oil of *Carum montanum*.

RI[a]	%[b]	Constituent
772	t	hexanal
823	0.6	*E*-hex-2-enal
833	0.1	*Z*-hex-3-enol
875	t	heptanal
923	0.1	α-thujene
930	0.9	α-pinene
964	0.2	sabinene
969	0.1	β-pinene
981	0.9	myrcene
1009	0.1	α-terpinene
1011	0.3	para-cymene
1020	0.6	limonene
1026	0.2	*Z*-β-ocimene
1037	0.7	*E*-β-ocimene
1048	0.8	γ-terpinene
1078	0.2	terpinolene
1081	t	nonanal
1100	0.1	undecane
1136	t	terpinen-4-ol
1224	t	methyl thymyl ether
1266	0.9	thymol
1275	t	carvacrol
1300	0.1	tridecane
1374	0.1	α-copaene
1379	0.5	β-bourbonene
1385	0.2	β-elemene
1413	2.5	β-caryophyllene
1430	0.8	α-bergamotene
1446	7.1	α-humulene
1468	0.4	ylangene
1471	2.2	germacrene-D
1483	0.4	myristicin
1486	0.2	α-selinene
1490	0.1	α-muurolene
1495	0.5	α-farnesene
1499	0.1	α-bisabolene
1512	0.6	β-sesquiphellandrene
1516	0.2	elemicin
1562	0.2	*E*-caryophyllene oxide
1589	8.5	dill apiole
1640	t	apiole
1738	62.8	nothoapiole
2069	0.1	osthole

[a]Retention indices on HP-1 column relative to C7-C22 *n*-alkanes.
[b]Area FID. t : trace compound (< 0.1%).

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Table 2. ^{1}H NMR, ^{13}C NMR and HMBC data of nothoapiole (1).

Atom no.	δ ^{1}H	multiplicity (*J* in Hz)	δ ^{13}C	HMBC (H → C)
1	-	-	118.8	-
2	-	-	145.1	-
3	-	-	134.6*	-
4	-	-	137.8*	-
5	-	-	133.4	-
6	-	-	136.7	-
7	3.77	s, 3H	60.2	C-2
8	5.90	s, 2H	101.3	C-3, C-4
9	3.94	s, 3H	60.5	C-5
10	3.89	s, 3H	61.6	C-6
11	3.33	dt (1.6 ; 6), 2H	28.4	C-1, C-2, C-6, C-12, C-13
12	5.95	m, 1H	137.8	C-1
13	4.97	m, 2H	114.5	C-11, C-12

Assignments deduced from COSY, NOESY, HMBC and HSQC experiments. *Assignments may be interchanged.

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Table 3. Antibacterial activities of *C. montanum* essential oil and nothoapiole (1)

Compounds/antibiotic tested	Inhibition zone (mm)[a]					
	EO	EO	EO	EO	**1**	Amp[b].
Concentration of compounds in DMSO (%)	100	50	25	12.5	100	(30 µg)
Amount of substance per disc (µg.10^2)	90	45	22.5	11.25	90	
Strains						
Escherichia coli	6	5	0	0	0	12
Enterococcus faecalis	0	0	0	0	NT	32
Pseudomonas aeruginosa	0	0	0	0	NT	30
Staphylococcus. aureus sub aureus	11	9	5	6	10	33
Staphylococcus epidermitis	16	14	10	7	8	62
Staphyloccus saprophyticus subsp.saprophyticus	0	0	0	0	NT	40
Staphylococcus simulans	28	26	17	15	8	32
Staphylococcus lugdunensis	12	6	0	0	9	9
Candida tropicalis	14	12	11	10	10	0

[a] Including diameter of the paper disc (6 mm). NT : Not Tested.

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